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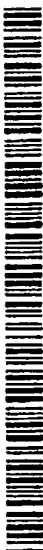


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(54) Title: HUMAN FMO3 GENE MUTATIONS AND POLYMORPHISMS, AND USES THEREOF

(57) Abstract: The present invention relates to human FMO3 gene polymorphisms and more particularly to uses thereof in the diagnosis of trimethylaminuria. There is provided a method for detecting an altered metabolism of a substrate of a flavin-containing monooxygenase form 3 (FMO3) enzyme in an individual, detecting a susceptibility of an individual to a substrate of the FMO3 enzyme in an individual, and for detecting a predisposition of an individual to a disorder associated with an exposure to a heteroatom-containing chemical compound, by detecting mutations or polymorphic variants of the FMO3-gene.

HUMAN FMO3 GENE MUTATIONS AND POLYMORPHISMS, AND USES THEREOF

Description of WO0123603

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HUMAN FMO3 GENE MUTATIONS AND POLYMORPHISMS, AND USES THEREOF BACKGROUND OF THE INVENTION (a) Field of the Invention

The invention relates to human FMO3 gene polymorphisms and mutations, and more particularly to uses thereof in pharmacogenomics and the diagnosis of inborn errors of metabolism such as trimethylaminuria and predisposition to hypertension.

(b) Description of Prior Art

Inter-individual variations resulting in population-wide differences in the metabolism of foreign compounds or xenobiotics may contribute to the susceptibility of humans to adverse chemicals or drug reactions and disease states. Most incidences of genetic variation can be accounted for by more prevalent alleles such as alleles with a frequency of greater than 1% in the general population, called polymorphisms or common variants. It is probable that common variants may contribute in a significant fashion to disease susceptibility. Pharmacogenomics allows for the identification of genetic variation in drug-metabolizing enzymes and the identification of individuals who will benefit most or least from a given medication.

Detoxification of xenobiotics including drugs, food additives and environmental chemicals is mediated by Phase I (oxidative) and Phase II (conjugative) reactions. The microsomal cytochrome P450 (CYP) family of monooxygenases is highly polymorphic in humans.

CYP3A4 is largely responsible for hepatic drug metabolism. However, CYP2D6 metabolizes a variety of widely prescribed drugs, and individuals with impaired CYP2D6 activity show a number of drug interactions

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resulting from aberrant metabolism due to inactivating polymorphisms.

Human flavin-containing monooxygenases (FMO) (EC 1.14.13.8) are microsomal NADPH-dependent flavoprotein enzymes that catalyze the oxygenation of nucleophilic nitrogen-, sulfur-, phosphorus- and other heteroatom-containing chemicals, drugs and pesticides.

The FMOs belong to the F5SP flavocytochrome c sulfide dehydrogenase subfamily of flavoenzymes, NAD(P)H-dependent monooxygenases and reductases. FMOs are membrane-bound proteins that have been detected in all secretory cell types that have been examined. Human FMOs are 532-558 amino acids in length, with specific amino acids highly conserved in all species, particularly residues 4-32 and 186-213, which contain the FAD and NADPH-binding domains, respectively (Cashman J (1995) Chem Res Toxicol 8: 166-181). The amino acid sequence identity between human isoforms is at a minimum of 52%. FMO enzymes have a broad substrate specificity compared to other mammalian monooxygenases, with at least 1,000 known substrates for these enzymes. They represent a family of five monooxygenases in mammals designated FMO, forms 1-5.

Differences in drug metabolism among animal species have been recognized for more than 50 years.

Human FMO drug metabolizing enzymes are thought to have evolved as a multi-gene family from an original monooxygenase such as that cloned and characterized from yeast (*Saccharomyces cerevisiae*) which has high amino acid sequence conservation with mammalian FMO.

Of the five FMO monooxygenase enzymes, FMO3 constitutes the prominent form that converts nucleophilic heteroatom-containing chemicals and endogenous materials to polar, readily excreted oxygenated metabolites, and this facilitates their

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elimination. FMO3 constitutes the major adult human hepatic isoform. Biogenic amines including primary amines, such as tyramine and phenylethylamine, and tertiary amines are good substrates for human FMO3 (Cashman J (1995) Chem Res Toxicol 8: 166-181).

Tertiary amine substrates include trimethylamine (TMA), antihistamines, and (S)-nicotine. TMA and tyramine are N-containing substrates of the enzyme.

Commonly used drugs such as tricyclic antipsychotics, cimetidine, ranitidine, albendazole and verapamil are also oxygenated by human FMO3 (Cashman J (1995) Chem Res Toxicol 8: 166-181, Ziegler, 1990).

The odorous, dietary-derived tertiary amine trimethylamine (TMA) is N-oxygenated by human FMO3 to the non-odorous trimethylamine N-oxide (TMANO). TMANO is excreted in a detoxication and deodorization process.

In normal humans, over 95% of TMA is metabolized to TMANO and is excreted in the urine at concentrations of less than 18 μ mol of TMA/mmol creatinine under normal dietary conditions. In a small number of humans, TMA is not efficiently metabolized to TMANO, and those individuals suffer from trimethylaminuria or fish-like odor syndrome, due to the presence of relatively large amounts of this odorous volatile amine in bodily excretions, including urine, sweat and breath.

Trimethylaminuria (TMAuria), first described in 1970, is an autosomal recessive inborn error of metabolism, which results in a partial or total inability to oxidize TMA to TMANO. (Treacy EP et al.

Hum Molec Genet 7: 839-845,1998) and a severe body odor reminiscent of rotten fish emanating in sweat, breath and urine with associated psychosocial disorders. This condition, previously thought to be rare, is now being increasingly detected in severe and

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milder presentations. TMAuria occurs in Australians and has a predicted incidence of 1/40, 000 in North Americans.

Current treatment strategies include dietary restriction of TMA precursors (choline, lecithin and carnitine), the intermittent use of antibiotics such as metronidazole, and other supportive measures.

Treatment options for this condition are limited and not well studied. Current strategies include limitation of choline and TMA precursors.

Dietary restrictions are insufficient for a number of individuals (related to genotype). Also some individuals over restrict choline which may also cause side-effects.

A number of individuals are consuming severely protein-restricted diets. Choline is an essential amine required for synthesis of phosphatidylcholine, sphingomyelin, acetylcholine and betaine (essential for remethylation of homocysteine) and for myelination. Choline deficiency causes memory impairment, liver and kidney dysfunction and cell death by apoptosis.

In 1998, choline was classified as an essential human nutrient by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences. Choline intake requires careful assessment in children and expectant mothers. There is as yet no studies of choline requirements in individuals with TMAuria on and off diet, and the precise choline limitation required for satisfactory decreases of TMA levels and relief of symptoms has never been studied.

Choline and derivatives such as phosphocholine are present in many food stuffs including milk and infant formulas in a substantial amount (25). Precise definition of choline requirements in individuals with

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TMAuria would be useful to determine a choline- restricted formula.

The structure for the FM03 gene has been characterized and maps to chromosome 1q23-25. The FM03 gene is approximately 28 kb in length, with 1 noncoding and 8 coding exons (Gene Bank Accession Number AL021026).

Although substrates are generally detoxicated by FMO-mediated metabolism, the FM03 enzyme has been implicated in the bioactivation of a number of xenobiotics (Cashman J (1995) Chem Res Toxicol 8: 166- 181). Thus, inactivating variants or polymorphisms of human FM03 may contribute to the pathophysiology of diseases and adverse reactions or exaggerated clinical responses to specific medications.

There exist genotyping assays which predict altered drug metabolism for the parallel phase I drug metabolizing enzyme system cytochrome P450.

There are at present no clinical tests available for pharmacogenomics with respect to FM03 and no markers for altered predisposition to handling of FM03 substrates.

It would therefore be highly desirable to be provided with a diagnostic test for trimethylaminuria.

It would also be highly desirable to be provided with identified pharmacogenomic polymorphisms of FM03. The detection of same would allow the determination of differential responses to medications and environmental toxins related to diseases such as those involving a complex pathophysiology. A large scale screening of the population with the detection of same would be advantageous to the pharmaceutical industry. The upregulation or downregulation of such polymorphic genotypes may also be made possible.

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SUMMARY OF THE INVENTION

One aim of the present invention is to provide a diagnostic test for trimethylaminuria (TMAuria).

Another aim of the present invention is to provide identified polymorphisms of FM03. The identified polymorphisms may allow the determination of differential responses to medications and environmental toxins causing diseases.

Mutations of the FM03 gene have been determined to cause trimethylaminuria.

Several single nucleotide polymorphisms (SNPs) of the FM03 gene have been identified. Rare mutations were discovered and identified use of the mutations and polymorphisms to determine their consequences for detoxication of drugs, chemicals and endogenous materials is also provided.

The distribution of the three polymorphisms V257M, E158K and E308G was studied in different combinations in Quebec individuals (Genetic Signatures). In vitro expression analysis in an E. coli system indicated that the codon 158 and 257 polymorphisms are pharmacogenomic, prevalent polymorphisms. Preliminary clinical evidence is provided to the effect that the E308G polymorphism, particularly when in cis with the variant codon 158 allele, alters oxidation of trimethylamine, likely applicable to other FM03 substrates. Analysis of cDNA expressed FM03-maltose-binding fusion proteins for two of those polymorphisms demonstrated altered N- oxygenation for 10- (N'-N-dimethylaminopentoyl)-2- (trifluoromethyl) phenothiazine, indicating that these are significant

pharmacogenomic polymorphisms.

Diagnostic tests for the mutations and polymorphisms are provided. The study of the distribution and population frequency of these three

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polymorphisms (pharmacogenomics) may likely predict adverse or altered response to typical FM03 substrates and common medications and indicate predispositions to complex disorders associated with disordered xenobiotic and biogenic amine metabolism. Expression analysis for the third polymorphism may be effected.

Loss of N-oxygenation and decreased clearance of amine-containing chemicals or drugs may contribute to adverse drug reactions, interactions with catecholamine or exaggerated clinical response to specific medications.

The detection of FM03 polymorphisms may be used to determine differential responses to medications and environmental toxins applicable to complex disease pathophysiology, and to determine high throughput screening for polymorphism detection (DNA chip technology) for large scale population, which may be advantageous to the pharmaceutical industry.

Polymorphism FM03 genotypes may also be upregulated or downregulated.

In accordance with the present invention, there is provided a method for detecting an altered metabolism of a substrate of a flavin-containing monooxygenase (FMO) enzyme or an isoform thereof in an individual. The method comprises detecting at least one of a mutation and a polymorphic variant of a gene encoding the FMO enzyme or isoform thereof in a sample from the individual, whereby the at least one of the mutation and the polymorphic variant is indicative of an altered metabolism for the substrate.

In accordance with the present invention, there is also provided a method for detecting a susceptibility of an individual to a substrate of the FMO enzyme or isoform thereof in an individual. The method comprises detecting at least one of a mutation

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and a polymorphic variant of a gene encoding the FMO enzyme or isoform thereof in a sample from the individual, whereby the at least one of the mutation and the polymorphic variant is indicative of a susceptibility to the substrate.

In accordance with the present invention, there is further provided a method for detecting a predisposition of an individual to a disorder associated with an (adverse) exposure to a heteroatom-containing chemical compound, an intermediate or a metabolite thereof associated with carcinogenesis or having a toxic, pro-carcinogenic or carcinogenic potential. The method comprises detecting at least one of a polymorphic variant and a mutation of a gene encoding the FMO enzyme or an isoform thereof in a sample from the individual, whereby the at least one of the polymorphic variant and the mutation is indicative of exposure to the chemical compound, the intermediate or the metabolite thereof.

In accordance with the present invention, there is further provided a method for detecting a predisposition to hypertension in a patient, the method comprising detecting at least one of a mutation and a polymorphic variant of a gene encoding the FMO enzyme in a sample from the patient, whereby at least one of the mutation and the polymorphic variant is indicative of a predisposition to hypertension.

In accordance with the present invention, there is further provided a method for the treatment of an individual having a disorder associated with an altered activity of the FMO enzyme or an isoform thereof. The method comprises supplementing the individual with riboflavin to increase the altered activity of the FMO enzyme or the isoform thereof.

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The mutation or polymorphic variant may inactivate partially or totally the activity of the FMO enzyme.

The isoform of the enzyme may consist of form 3(FM03).

The polymorphic variant may comprise a polymorphic variant from the group consisting of E158K, V257M and E308G, and the mutation may comprise a mutation from the group consisting of P153L, E305X, M66I, E314X, R492W, A52T and R387L.

The altered metabolism may be associated with an idiosyncratic reaction to the substrate.

The altered metabolism may be associated with a disorder such as a cancer. The disorder may also be TMAuria.

The substrate may consist of a xenobiotic or an endogenous material relative to the individual. The xenobiotic may be a drug, a food additive, a pesticide, a plant toxin, an organic chemical compound or an aromatic amine. The substrate may be a biogenic amine contained in the individual's diet. The biogenic amine may consist of a tertiary amine, such as trimethylamine (TMA), tyramine or catecholamine.

For the purpose of the present invention, the following abbreviations are defined below.

"FMO" is intended to mean human flavin-containing monooxygenase; "FM03" is intended to mean human flavin-containing monooxygenase form 3; "FM01" is intended to mean human flavin-containing monooxygenase form 1; "TMA" is intended to mean trimethylamine; "TMANO" is intended to mean trimethylamine N-oxide; "5-APT" is intended to mean 10-(5-Aminopentyl)-2-(trifluoromethyl) phenothiazine;

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"5-DPT" is intended to mean 10- (N, N-Dimethylaminopentyl)-2- (trifluoromethyl)phenothiazine; MBP: maltose binding protein; "FM03 MBP" is intended to mean human flavin-containing monooxygenase form 3 maltose binding protein; "SDS-PAGE" is intended to mean sodium dodecyl sulfate polyacrylamide gel electrophoresis; "TCA" is intended to mean trichloroacetic acid; and "TMAuria" is intended to mean trimethylaminuria.

A "polymorphism" is intended to mean a nucleotide substitution of a gene that occurs at a frequency greater than 1% in individuals in the general population.

"Severe" TMAuria is intended to mean a reduction of TMA oxidation below 50% of normal oxidation.

A "genetic signature" with respect to a substitution is intended to mean a combination of a polymorphism or a mutation identified in an individual or haplotype for the substitution of interest.

The term "patient" is intended to mean a mammal preferably human.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates in a diagram the structural organization of FM03cDNA.

DETAILED DESCRIPTION OF THE INVENTION

Two mutations of the FM03 gene, P153L and E305X (OMIM accession numbers 136132.001 and 136132.004) account for greater than 90% of cases of TMAuria in Australians. Six other mutations (Fig. 1) and three intragenic polymorphisms (E158K, V257M and E308G) which appear to affect TMA and other FM03 substrate oxidation are also reported. In a North-American

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cohort (including 4 Canadians) 100% ascertainment of mutant alleles was reported and in some probands, the second mutant allele comprises two of these polymorphisms in cis, (K158-G308) (Akerman BR et al.

Am J Hum Genet (1997) Supplement to vol 61 (no. 4).

Abs. no. 281) (Table 1). Of the three polymorphisms identified in Quebec (see Table 2), expression of mutant cDNAs for the codon 158 and 257 polymorphisms showed altered N-oxygenation for 5-DPT (a phenothiazine substrate), tyramine and TMA, suggesting that these polymorphisms are significant prevalent pharmacogenomic polymorphisms.

A cohort of individuals was ascertained in North America with severe TMAuria.

Of 70 referrals for a suspected diagnosis of TMAuria, TMAuria was confirmed in 20 individuals with reduced TMANO/TMA ratios. (Normal values are: TMANO/TMA relative% ratios > 98; 2). Among these, some had symptoms of hypertension and migraine with abnormal dopamine metabolism, suggesting that mutations of the FM03 gene may result in abnormal catecholamine metabolism.

Four new FM03 mutations were detected in this cohort: two missense (A52T and R387L), and one nonsense (E314X) (Akerman BR et al. Am J Hum Genet (1997) Supplement to vol 61 (no. 4). Abs. no. 281). The fourth allele is apparently composed of two relatively common polymorphisms (K158-G308) found in the general population.

A number of prevalent nucleotide polymorphisms of the human FM03 gene were identified. Polymorphisms that are prevalent in French Canadian and Australian populations were studied. The cDNA-expression analysis for two of these prevalent human FM03 polymorphisms showed altered N-oxygenase activities, indicating that

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these are significant pharmacogenomic polymorphisms.

Two prevalent polymorphisms of this gene (E158K and V257M) modulate the activity of human FM03. These polymorphisms are widely distributed in Canadian and Australian Caucasian populations. In vitro analysis of wild-type and variant human FM03 proteins expressed from the cDNA for these two naturally-occurring polymorphisms showed differences in substrate affinities for nitrogen-containing substrates. Thus, for polymorphic forms of human FM03, lower k_{cat}/K_m values for N-oxygenation of 10- (N, N-dimethylaminopentyl)-2- (trifluoromethyl) phenothiazine, trimethylamine and tyramine were observed. The results imply that prevalent polymorphisms of the human FM03 gene likely represent low penetrance predispositions to diseases associated with adverse environmental exposures to heteroatom-containing chemicals, drugs and endogenous amines.

Analysis of polymorphisms may be performed by PCR testing in Quebec residents with application to metabolism of FM03 substrates (medications) in humans that are heterozygous or homozygous for polymorphisms.

The genotyping methods are described herein. The genotypes observed in Quebec (Genetic Signatures) and methods thereof are also described. Deficient human FM03 N-oxygenase activity has implications for the abnormal metabolism of many other endogenous dietary and medicinal amines.

We previously reported that the enzyme encoded by the human FM03Glu158 polymorphic allele is more active for the tertiary amine substrates TMA and 5-DPT than the enzyme encoded by the less prevalent human FM03Lys158 allele (Treacy EP et al. Hum Molec Genet 7: 839-845, 1998).

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Two new other polymorphisms are provided herein. The presence of these prevalent FM03 polymorphisms implies that some individuals may be more susceptible to the effects of environmental chemicals and to idiosyncratic drug reactions. Thus, if a drug or chemical is dependent on human FM03 for detoxication and if an individual possesses a impaired polymorphic form of FM03 then the drug or chemical may produce an exaggerated clinical response and this could lead to adverse reactions.

Human FMO1 is not functionally active in adult human liver (Cashman J (1995) Chem Res Toxicol 8: 166- 181). Based on the kinetic parameters observed, human FMO1 does not appear to make a significant contribution to the metabolism of TMA. From the present results it is likely that human FMO1 does not rescue an individual that is deficient in FM03 from suffering the consequences of trimethylaminuria. Human FMO1 does not significantly N-oxygenate primary amines such as tyramine or 5-APT but can participate in the N-oxygenation of chemicals or drugs containing the tertiary amine functionality.

From the in vitro data, the codon 257 polymorphism appears to show substantial differences in kinetics for the biogenic amine substrate, tyramine and the dietary amine, TMA. This may have clinical consequences.

The maintenance of the prevalent codon 158 polymorphism in the Caucasian populations examined may not result solely from genetic drift, but perhaps as a consequence of molecular drive, whereby particular polymorphisms with selective advantages persist, for example, to combat exposure to plant toxins in particular geographic regions. The distribution of the two codon 158 alleles is almost in equilibrium in

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these populations, suggesting that this may be an older polymorphism that is now balanced. Human FM03 V257M is of lower prevalence and may represent a founder effect.

Variation in human drug metabolism by genetic polymorphisms may increase the risk for acquiring exposure-related disease, including cancer, with important public health consequences. Generally, oxidative metabolism of heteroatom-containing compounds by CYP-dependent processes leads to production of chemical intermediates with increased potential for toxic or carcinogenic properties.

Although human FMOs generally convert lipophilic heteroatom-containing compounds to polar, readily excreted oxygenated metabolites that possess decreased toxic potential, they may also catalyze the N- oxygenation of a wide array of xenobiotics such as plant toxins, organic chemicals and aromatic amines associated with carcinogenesis (Cashman J (1995) Chem Res Toxicol 8: 166-181).

Consistent with the fact that human drug- metabolizing enzymes have endogenous substrates and are prevalent not as neutral balanced polymorphisms but for their selective advantages, it was previously shown that human FM03 metabolizes biogenic amines such as tyramine and phenethylamine resulting in great stereoselectivity of their oxime metabolites.

Formation of oxime metabolites generally terminates the pharmacological activity of the parent amine (Lin, J et al. (1997) Chem. Res. Toxicol. 10: 842-852). It is shown herein that the methionine variant at codon 257 of human FM03 shows decreased N-oxygenation for the substrate tyramine. Tyramine is an indirectly- acting sympathomimetic that exerts its pressor effect through amine uptake into the sympathetic nervous

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system with release of norepinephrine from synaptic vesicles. It is thus possible that human FM03 polymorphisms affecting tyramine or other biogenic amine metabolism may predispose humans to variable tolerance to tyramine or other biogenic amine- containing foods and associated symptoms.

It is shown that human FM03 null alleles are rare and cause a severe phenotype with lack of oxygenation of human FM03 substrates such as TMA (Cashman JR et al. Chem Res Toxicol 10: 837-841,1997; Treacy EP et al. Hum Molec Genet 7: 839-845,1998). On the basis of the in vitro data described herein, the human FM03 polymorphisms have been shown to have milder effect on N-oxygenation activity. The polymorphisms are, however, of higher frequency, and thus they are more likely to show specific associations with complex diseases in populations.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

TMAuria is caused by mutations of the FM03 gene in a North American Cohort Urine detection

Early morning first void samples were collected and acidified with NHC1 to pH 2 upon collection from subjects, and TMA and TMANO were measured by fast atom bombardment mass spectrometry using ¹⁶N-labeled TMA and TMANO as internal standards, a highly sensitive assay.

Values were expressed as mmole TMA or TMANO per mole creatinine. Control ratios for 20 adult urine collected from volunteers were established as TMANO: TMA percentage ratios >98: 2%.

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Mutation detection

DNA was extracted by standard methods from whole blood collected in EDTA from probands with TMAuria. The samples were then screened by PCR and restriction digestion for amino acid polymorphisms previously identified

(E158K, V257M and E308G) and for mutations detected in the Australian TMAuria cohort (M661, P153L, E305X, R492W).

Table 1 lists conditions used for the restriction diagnostic tests performed and the relevant primer sequences.

Table 1

Diagnostic PCR Reactions for human HFM03 Variants Allele Exon Primers (sense-antisense ; 5'to 3') SEQ Restriction Expected Pattern

ID No: enzyme normal (bp) mutant(bp)
A52T 3 2038:GACCTGATCAGTATACTCATTTA 1 NheI 79 147
2025:AATGGGAAGTCTGGGAAACACTT 2M661 3 As above MseI 147 122

25
P153 4 2055: TTGTTCCGGACATCATGTGTAGC3 AluI 263 263
Gen2:TCCCTGCTGTGGAAGCATTT 4 66 45

21
K158E 4 As aboveHinn 253 217
76 76

36 V257M 6 C1 :TTCCAGAAGTGCTCCTGGG 5Hsp9211 97 72
2005:GCTTGAATCTTGCAATTCATCTGC 6 60 60

25
E305X 7 2019:CCTTATCAATTATATATGGACC 7EcoRI 365 529
2016:GGACCTTGTAAGTAGGATTATTG 8 164

E308G 7 As above BsaI 362 529
167

E314X 7 As above AclI 409 532
122

R387L 7 As above MseI 112 102102 88 R492W 9 2049:GAAATGCCATACTGACCCCATGG 9BstXI 24
2050:TAGCAAAGCCCCGTCTGGGTAT 10 211 186

25

To confirm the three newly identified mutations, restriction enzyme analysis was performed on three independent PCR products from the probands.

For the A52T and R387L alleles, the mutations ablated naturally-occurring restriction sites, while the E314X variant created a new restriction site, as shown in

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Table1. The mutation assays were subsequently used to determine the frequency of mutations in control samples from the province of Quebec, Canada.

Results

Of 28 individuals referred for investigation of TMAuria, we identified 10 who had a marked decrease in TMA oxidation, with less than50% of the total TMA in the N-oxide form, as shown in (Table 2). Control ratios for these compounds were defined as being greater than 98: 2% in our laboratory. Further analysis was possible for 10 of the remaining undiagnosed consultants by gas chromatography-mass spectrometry; no abnormalities were detected. In addition, testing of these 10 urine samples by NMR spectroscopy ruled out dimethylglycinuria, a recently described entity which also presents with a fish-like odor and mild TMAuria.

Four of the parents of our defined TMAuria probands (obligate heterozygotes) were also tested for TMANO: TMA ratios. For one heterozygote carrying the E314X mutation the TMANO: TMA ratio was noted to be 95: 5. For the other heterozygotes carrying missense mutations the values obtained (without a TMA challenge) were observed to be in the normal range (data not shown).

Of the 10 probands with perturbed TMANO: TMA ratios, DNA was available from 8 individuals; mutation analysis was performed on these samples. The ethnic origins as well as the FM03 genotypes of these are detailed in Table 2: 7 of the probands are of European Caucasian origin; one is Metis (French Canadian/North American Indian).

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Table 2

FM03 Mutations and Phenotypes in Eight Probands with TMAuria

Biochemicalphenotype Genotypee Subject Agea EthnicTMANOt TMAb TMANO : Nucleotide changeAmino acid (sex) origin TMA

ChangeId (M)55 Irish/ 33. 90 50.71 39: 61458G>T- (472G)/ P153L- (E158)/

English458G>T (472G) P153L-(E158) 2 (F) 28 German/17.86 22.73 44: 56154G>A- (472A)- A52T- (K158)-

German 923A>G/ E308G/(K158)-

Irish/ (472A)-923A>G E308G

French 3 (M) 23 German/6.05 66.2 8.4: 91.6458G>T- (472G)/ P153L- (E158)/

Russian (472G)-1923A>G(E158)-E308G-

E314X4 (M) 12 German 11.31 60.24 16: 84458G>T- (472A)/ P153L- (K158)/ 458G>T- (472A) P153L- (K158)5c (M) 10 Metis 5.47

65.34 8: 92(472A)-1160G>T/ (K158)-R387U (472A)-1160G>T (K158)-R387L 6 (M) 11 English 6.93 51.90 12: 88458G>T/(472G)/

P153L- (E158)/ (472G)-913G>T (E158-E305X 7 (F) 8 French 12.20 75.8 14: 86 458G>T/1474C>T/P153LR492W;

Canadian (472G/A) 8 (M) 2 English 8.36 52.96 14: 86 458G>T (472G)/ (158

458G>T (472G) E/K P153L (E158) aAge at diagnosis. bUnits are mmol TMA or TMANO/mol creatinine. cIndicates the presence of

migraine. dIschemic heart disease.e In addition to the mutations, the genotype at polymorphic codon 158 is given in parentheses. f

Normal values: TMANO > 98% total, TMA < 2% total.

Of previously described mutations, P153L was found to account for nine alleles in this study, while the E305X and R492W mutations

were each observed uniquely. M66I, previously observed in the Australian cohort, was not detected in this study. Four new disease-causing alleles were detected: A52T (c.154G#A), E308G (c.923A-G), E314X (c.940G#T), and R387L (c.1160G#T). The substitutions A52T, E314X, and

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R387L were not detected on screening 60 control chromosomes from French Canadians. The E308G allele was found to segregate in two probands, homozygous in one compounded with another missense allele (A52T).

The G308 allele was detected in 36/198 normal control chromosomes from a Quebec francophone population (q=0.18) and in 18 of 118 chromosomes of a Quebec anglophone population (q=0.15). We observed E308G in the homozygous state in one French Canadian control.

The allele frequency of the G308 allele in probands (0.19) is not statistically different from the background frequency.

Thus, 10 cases of severe TMAuria were confirmed in 28 North Americans with the presenting symptom of malodor. The results are similar to those of the group of George Preti in Philadelphia, who reported that one-third of their referrals for the same complaints were due to TMAuria. While our report defines cases of severe TMAuria, it does not address the possible phenotype of heterozygotes or very mild cases which may be detected by TMA or choline challenge testing.

All of the probands studied have TMA oxidation of less than 50% of normal.

A relationship between ablation of FM03 activity and symptoms suggestive of disordered biogenic amine metabolism such as hypertension has been noted. In this study, 3 to 10 TMAuria probands also exhibit "labile" hypertension according to their physicians, although one of these individuals (Table 2, Subject 1) also has ischemic heart disease which may cause secondary hypertension. Three of the 10 confirmed subjects report the symptoms of classical migraine, a condition noted to be associated with disordered metabolism of biogenic amines such as tyramine. As FM03 is expressed in brain and

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metabolizes biogenic amines, this association merits further study.

It is now established that mutation of the FM03 gene causes severe TMAuria in North American probands.

100% ascertainment of mutations in cases defined by the admission criteria of the study are reported. Our previous studies of Australian TMAuria probands detected two relatively common mutations (P153L and E305X) in probands of British origin and two rare alleles (M66I, R492W). In this North American cohort, it also found that the mutation P153L is relatively frequent, accounting for 9 of 16 mutant chromosomes.

The segregation of the alleles E305X and R492W was also noted. These alleles may have originated in the British Isles.

Four new mutations were detected. The alleles A52T, E314X, and R387L may be "private" mutations, as they have been observed once in the probands of differing ethnic backgrounds. Loss of activity is strongly predicted from truncation of the FM03 protein at codon 314 since we know that deletion of even the final 30 amino acids of this 582 residue protein will ablate function in vitro (Cashman J (1995) Chem Res Toxicol 8: 166-181). While the mutations A52T and R387L have not been expressed, they satisfy several conditions for designation as disease-causing: the changes have not been observed in controls (n=30), they are nonconservative substitutions, and no other changes were identified in the probands on sequencing all expressed FM03 exons. The A52 and R387 residues appear to be highly conserved within the FMO gene family, as seen in Table 3.

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Table 3

Amino Acid Sequence Surrounding Human FM03

Codons 52 and 387; Identity and Variation among Other FMO Proteins

HFM03a partial amino acid sequence Enzyme A E E G R A52S IY K Reference RbFM03 @ ***** Q (35) MFMO3 @ *****
 ** (36) GpFM05 P * (37) HFM05 P * (37) HFM01 V ***** L ** (38) MFMO1 V ***** L ** D16215b PFM01V L (39) RbFM01V L
 N (40) GpFM01 V * D ***** (41) RhFM02 V * D ***** Q U59453bHFM04S K D M T R V (42) RbFM04 S K D M T R V W (35)
 HFM03a partial amino acid sequence Enzyme V D L QS R387 W A A Q Reference RbFM03 T A (35) MFMO3 T A (36) GpFM05 S E *
 * G *** V * (37) HFM05S E G T (37) HFM01 G E T * A *** V R (38) MFMO1 G E T * A *** V V * D16215b PFM01G' TA AV R (39)
 RbFM01 G E T * A *** V V * (40) GpFM01 * E *** A *** T R (41) RhFM02 A E *** A *** V T R U59453bHFM04 T E *** A *** V T R (42)
 RbFM04 T E *** A *** T R (35) a Abbreviations : Gp, guinea pig; H, human; M, mouse; P, pig; Rb, rabbit; Rh, Rhesus monkey; Rt, rat. b GenBank Accession Number.

A comparison of amino acid sequences surrounding these residues in 12 published FMO sequences indicates that R387 is conserved in all 12 while the alanine at codon 52 is conserved in 10 of these 12. The variation at codon 52 seen in the 2 published FM04 sequences may contribute to substrate specificity differences seen between FMO isoforms.

The G variant at codon 308 was observed in two probands with TMAuria. Subject 2 is an E308G homozygote with a single copy of the A52T missense mutation; Subject 3 is a compound heterozygote, with

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E308G and E314X on one homologue and P153L in trans (Table 2). The G308 allele was originally observed segregating in normal controls, and it exists as a frequent polymorphism in the Quebec population (q=0.18). The E308G polymorphism may also mediate

other variations of drug and chemical detoxication.

The genotype for Subject 2, who has had complete sequencing of all FM03 coding exons, is T52-K158-G308 on one allele and A52-K158-G308 on the other.

Previous in vitro expression studies on the codon 158 polymorphism indicated the K158 form of the protein is a poorer TMA N-oxygenator than the E158 form. The findings demonstrate that the G308 change on the K158 background functions as a TMAuria mutation. It is possible that these two common changes in cis further diminish the activity of the enzyme and render it incapable of compensating for the A52T mutant, resulting in a severe TMAuria phenotype.

As shown in Fig. 1, mutations appear to cluster in exon 7, with three of the four identified changes in this exon occurring within a 10 amino acid "hot spot". These changes fall near or in the areas of high homology described by Ziegler. They are in close proximity to the FATGY signature found in all the mammalian enzymes, while the R387L mutation is found in an area highly conserved among FMOs, strongly suggesting that this substitution affects protein function.

Each of the eight genotypically defined probands studied was ascertained in a different region of North America; presumably these regions differ in background carrier frequencies for FM03 mutations.

Since one of the probands is from Quebec, the overall population frequency question was addressed by screening for known mutations (M66I, P153L, E305X,

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R492W) in a Quebec control group. Of 320 control chromosomes, only one carrier for the mutation E305X was detected. Although the number surveyed is small, this predicts that severe TMAuria should be rare in Quebec. Milder variations that may manifest with a precursor load may be more common. This condition may "cluster" in specific regions of the United States and Canada due to "founder effect" and "genetic drift", as occurs in the Melbourne region of Australia. In addition to possible evolutionary advantages in detoxication, there are other hypotheses to account for the maintenance of mutant FM03 alleles in the population. These must include other possible selective advantages of heterozygosity, perhaps in blood pressure homeostasis or in metabolism of choline, now recognized to be a vital amine involved in many pathways of intermediary metabolism

EXAMPLE II

Population Specific Polymorphisms of the human FM03 gene:

Significance for Detoxication Study Population

The human FM03 polymorphisms E158K (c. 488 G-A) and V257M (c. 769 G-A) were previously identified in a cohort of individuals with trimethylaminuria and normal controls from Melbourne, Australia using single stranded conformational polymorphism (SSCP) screening and sequencing of the FM03 gene (Treacy EP et al. Hum Molec Genet 7: 839-845, 1998). Following institutional ethics approval, the frequency of these substitutions were determined in 170 normal control individuals from Quebec (i. e., 110 Francophones and 60 Anglophones) and 50 normal controls from Victoria, Australia. Genomic DNA was prepared from lymphocyte preparations using

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standard procedures. Amplified DNA fragments obtained by the polymerase chain reaction were subjected to restriction enzymatic digestion, and visualized by ethidium bromide staining after agarose or polyacrylamide gel electrophoresis. Table 1 illustrates the conditions used for each diagnostic assay.

Table 4

Diagnostic PCR methods created to screen for identified human FM03 sequence changes among healthy controls Allele

Oligonucleotide Sequence SEQ ID Restriction Normal Variant NO : Enzyme Restriction Restriction

Pattern (bp) Pattern (bp) E158K 2055: 5'-TTG TTC CGG ACA TCA TGT GTA GC-3'3' HinfI 253 217

Gen2: 5'-TCC CTG CTG TGG AAG CAT TT-3'4 76 76

36 V257M C1 : 5'-TTC CAG AAG TGG CTC CTG GG-3'5 Hsp9211 97 72

60 60

20055'-GCT TGA ATC TTG CAT TCA TCT GC-3'6 25

Table 5 Genotype frequencies for the human FM03 polymorphisms

E158K and V257M in Quebec, Canada and Victoria,

Australia

E158K V257M QUEBEC FRANCOPHONE POPULATION N=109 N=108

EE: 38 W: 101

EK: 56 VM: 6

KK: 15 MM: 1 QUEBEC ANGLOPHONE POPULATION N=60 n=58

EE: 16 W: 55

EK: 30 VM: 3

KK: 14 VICTORIA N=39 N=61

EE: 12 W: 50

EK: 20 VM: 9

KK: 78 MM: 2 Data Analysis

The statistical significance of the frequency of the human FM03 E158K and V257M polymorphisms between Canadians (i. e., Anglophones and Francophones) and Australians was calculated. A chi-squared analysis

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was used for human FM03 E158K to test the hypothesis that the proportion of chromosomes was not significantly different between the three groups of English Canadians, French Canadians and Australians (% squared, 2df = 2.52, p = 0.28). The Mantel-Haenszel test for small sample size was used for human FM03 V257M. There was no significant difference observed between the presence of the variant alleles in the three cohorts (X squared, 2df = 4.71, p = 0.095).

Chemicals[3H]-Tyramine was obtained from American Radioactivity Company (St. Louis, MO) and[14C]-TMA was obtained from Sigma Chemical Co., (St. Louis, MO). All chemicals and reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) in the highest purity commercially available.

Synthesis 10- (5-Aminopentyl)-2- (trifluoromethyl) pheno- thiazine (5-APT), its hydroxylamine and cis and trans oximes were synthesized by a modification of the procedures previously described. The tertiary amine 10- (N, N-dimethylaminopentyl)-2- (trifluoromethyl) pheno- thiazine (5-DPT) and its N-oxide was also synthesized by a procedure similar to the one previously described. The hydroxylamine and cis and trans oximes of tyramine were synthesized as previously described (Lin, J et al. (1997) Chem. Res. Toxicol. 10: 842- 852). cDNA-expression and Substrate Analysis
The human FM03 cDNAs were expressed as maltose binding fusion proteins. Site-directed mutagenesis for the human FM03 substitutions E158K and V257M were performed as previously described (Treacy EP et al.

Hum Molec Genet 7: 839-845,1998, Cashman JR et al.

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Chem Res Toxicol 10: 837-841,1997). For purposes of comparison, acDNA construct for the truncation variants E305X (previously reported) and 510X were also prepared as the maltose binding protein fusions.

Subcloning Human FMO1 and FMO3 cDNA into the Maltose Binding Protein Fusion Expression System

Human FM03 or FMO1 cDNA was inserted into the expression vector pMAL-c2 and PCR amplification was done in a fashion that allowed for fusion of human FM03 or FMO1 cDNA at the 3'-end of sequences encoding the maltose binding protein (MBP) as previously described (Treacy EP et al. Hum Molec Genet 7: 839- 845,1998). Each cDNA was individually cloned and confirmed by oligonucleotide sequencing of both strands. The creation and cDNA-expression of wild type Glu 158 and the common polymorphic form Lys158 FM03- MBPs has been previously described (Treacy EP et al.

Hum Molec Genet 7: 839-845,1998, Cashman JR et al.

Chem Res Toxicol 10: 837-841,1997). Another polymorphic form of human FM03 (i. e., Met 257) was created by oligonucleotide-directed mutagenesis and PCR in a similar fashion as described before. The truncation mutations of FM03-MBP (E305X and 510X) were created by PCR. The template was the pMAL-2c wild type human FM03 Glu 158 expression plasmid. At the appropriate position, the forward PCR primer contained a BamHI site that was followed by ATG. The reverse primer changed the codon following the site of truncation to an ochre stop codon, that was also part of a HindIII site. The truncated human FM03cDNA was synthesized using Taq polymerase under standard conditions. The PCR fragment was gel purified, digested with BamHI and HindIII and inserted with DNA ligase into the pMAL-c2 vector cut with the same restriction enzymes. Each desired truncation product

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was transformed into competent JM109 E. coli and plated onto LB-Amp plates. DNA isolated from colonies were shown to contain the desired truncation mutation by sequencing of both strands. The truncation expression plasmids introduced into bacterial strain JM109 were purified by affinity chromatography as previously described.

Electrophoresis and Immunoblotting

Overproduction of the affinity-purified human FM03-MBP fusion proteins and truncation variants was shown by fractionation on 12% sodium dodecyl sulfate polyacrylic gel electrophoresis (SDS-PAGE).

Immunoblots were done according to a previously described procedure using an affinity purified rabbit polyclonal antibody that was directed against the wild type human FM03-MBP fusion protein.

Enzyme Assays

Assay and analysis of human FM03-MBP fusion protein and analysis of N-oxygenation activity for the variant enzymes was done by a procedure described previously. The N-oxygenation 5-APT was done using an HPLC method essentially identical to the one described previously for a closely related compound. TMA and tyramine N-oxygenation was determined using radiometric assays described below.

For[3H]-tyramine and[14C]-TMA, incubations were carried out in 13 x 100 screw-cap culture tubes suspended in a 37 C water bath-metabolic shaker apparatus. The reaction mixture consisted of 10-30 ug of human FM03-MBP or variant, 0.05 M potassium phosphate buffer (pH = 8. 4), 0.8 mM diethylenetriaminepentaacetic acid (DETAPAC), 0.5 mM NADP+, 0.5 mM glucose-6-phosphate and 1 IU of glucose- 6-phosphate dehydrogenase in a total volume of 0.25 ml. The reaction was initiated by the addition of

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radiolabelled substrate to an ice-cold previously equilibrated enzyme solution, capped and incubated at 37 C with constant shaking. For TMA, the reaction was stopped by the addition of 0.25 ml cold CH₃CN containing 0.01 ml trichloroacetic acid (TCA). For tyramine, the reaction was stopped by the addition of 0.25 ml cold MeOH. The incubation mixture was thoroughly mixed and centrifuged and an aliquot was applied to the loading zone of a Whatman Diamond LK6DF TLC plate (Clifton, NJ) previously co-spotted with authentic starting material or products of the specified reaction. For TMA, after air drying, the plate was developed in methanol/chloroform/20% TCA (90: 10: 0.5, v: v). The following bands (R_f) were visualized by treating with iodine and scraped into scintillation vials for counting: TMA N-oxide (0.32) and TMA (0.08). For tyramine, after air drying, the plate was developed in methanol/dichloromethane/formic acid (20: 80: 0.2, v: v). The following bands (R_f) were visualized by UV-vis and scraped into scintillation vials for counting: cis and trans phenylacetone oximes (0.84), tyramine hydroxylamine (0.35) and tyramine (0.04). Each vial was counted in a Beckman LS-2000 scintillation counter and the percent product formation was computed from the data for calculation of the kinetic parameters.

FAD determination

A 0.5 ml aliquot of protein was combined with an equivalent amount of cold acetonitrile, mixed and centrifuged at 12,000 x g. The pellet was washed three times with cold acetonitrile. To the protein pellet was added 50 μ l of trifluoroacetic acid, mixed thoroughly and heated in a sealed tube at 55 C for at least 40 min. At the end of the reaction 100 μ l of cold acetonitrile was added as well as a sufficient

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amount of NH₄OH to neutralize the reaction. A portion of the mixture was injected directly onto a Hitachi HPLC system using a Rainin Microsorb MV C-18 reverse phase column (Rainin, Emeryville, CA). An eluent of water (containing 0.1% HClO₄) for the first 20 min followed by CH₃CN (containing 0.1% HClO₄) thereafter was used to separate FAD from other minor impurities by HPLC. FAD eluted during the first phase of the gradient and was detected at 450 nm with a retention time of 9.2 min. The amount of FAD was determined from a comparison of peak heights from a standard curve of FAD.

RESULTS Genotype Frequencies

The genotype frequencies for the two prevalent human FMO3 polymorphisms E158K, and V257M in healthy populations from Quebec, Canada and Victoria, Australia are listed in Table 5. On the basis of statistical analysis, the distribution of the two codon 158 polymorphic variants were found to be similar in Quebec Francophones and Anglophones and in the Australian population. Although the numbers were small, the methionine variant involving codon 257 appeared to be at higher frequency in the Australian population compared with the Quebec population.

Substrate N-Oxygenation

Previous studies showed that non-transformed host bacteria or bacteria transformed with pMal alone did not contain any detectable human FMO activity when grown in the presence or absence of IPTG. Previously, it was shown that the relative activity of human FMO3 MBP and some variants showed varying degrees of TMA N- oxygenation activity (Treacy EP et al. Hum Molec Genet 7: 839-845,1998, Cashman JR et al. Chem Res Toxicol 10: 837-841,1997). To examine this point more

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carefully and to quantify differences among the cDNA- expressed alleles, Km and Vmax values were obtained from double reciprocal plots of velocity versus substrate concentration. For the substrates examined, the formation of tertiary amine N-oxide, hydroxylamine or oxime metabolite was a linear function of protein concentration and with incubation time for at least 10 min. As shown by the kinetic constants listed in Table 4, wild-type human FMO3 MBP efficiently N-oxygenated 5-DPT, TMA and tyramine. Activities for human FMO1 MBP (a fetal hepatic FMO isoform that is expressed in adult kidney and intestine but not in adult liver) were evaluated in a similar kinetic fashion. The kinetic constants are listed in Table 6 for comparison. Human FMO1 MBP N-oxygenated 5-DPT with a very low Km value. In contrast, TMA N-oxygenation was significant but the concentration of substrate required for half-maximal activity of FMO1 MBP was 15- fold greater than that of human FMO3 MBP. The Km for human FMO1 MBP N-oxygenation of TMA compared favorably with that of TMA N-oxygenation for pig FMO 1 (CashmanJ (1995) Chem Res Toxicol 8: 166-181).

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Table 6

Kinetic Constants for Human FMO1 MBP, FMO3 MBP, and Variants of Human FMO3 MBP

Enzyme Km kcat(min⁻¹) Vmax/Km Substrate

Human FMO1 (n=6) 8.0#2. 4 4.0#1. 6 0.5 5-DPT

Human FMO3(WT) Glu158 (n=3) 154.931. 8 50.71. 6 0.33 5-DPT Lys158 (n=3) 187.534. 1 44.4 17. 7 0.23 5-DPT

Met257 (n=3) 82.3#41. 7 13.24. 6 0.16 5-DPT 510X (n=4) 61.6#12. 9 0.09#0. 09 0.001 5-DPT

Human FMO1 (n=4) 486 34.8 25.93. 7 0.05 TMA

Human FMO3(WT) Glu158 (n=4) 32.3#7. 1 189

human FMO1 MBP were 18-, 249-and 25-fold lower, respectively, than the Vmax/Km ratios for the wild type human FMO3 MBP enzyme.

Previously, we have examined the truncation mutation human FMO3 MBP 305X (Treacy EP et al. Hum Molec Genet 7: 839-845,1998). No detectable N- oxygenase activity was observed for 5-DPT, TMA and tyramine. For comparison, a human FMO3 cDNA construct that was truncated at codon 510 was also analyzed for substrate N-oxygenation activity with 5-DPT, TMA and tyramine. The Km values for FMO3 MBP 510X were similar to the Km values observed for FMO3 MBP Met 257, but generally, the Vmax values were significantly lower than the wild type fusion protein (Table 6).

The Glu 158 wild type human FMO3 MBP enzyme N- oxygenated 5-APT (i. e., the primary amine analog of 5- DPT) with a rate of 117 nmol/min/nmol of protein. As determined by HPLC, the major product was the hydroxylamine. A minor amount of oxime was formed and the ratio of cis : trans oxime was 79: 21. Human FMO3 MBP Lys158 N-oxygenated the primary amine 5-APT with a rate of 69.1 nmol/min/nmol of protein. As determined by HPLC, the major product was the hydroxylamine. A minor product was the oxime and it was formed in a ratio of cis : trans oxime 81: 19. By comparison, the human FMO3 Met 257 enzyme N-oxygenated the primary amine 5-APT with a rate of 28.1 nmol/min/nmol of protein. As determined by HPLC, the major product was the hydroxylamine. A minor product was the oxime and it was formed with a cis : trans stereoselectivity of 80: 20. Human FMO1 MBP did not significantly N-oxygenate tyramine or 5-APT.

Because 5-APT was present in large excess, the rates were determined at saturating substrate concentration and represent apparent Vmax values. We

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examined a number of human FM03 MBP truncation mutations introduced between codon 305 and the wild type length. There was no significant N-oxygenase activity observed for the humanFM03cDNA construct with a truncation less than codon 510. Similar experiments have been conducted with pig FMO1 MBP and a similar structure-function relationship has been observed (Cashman J (1995) Chem Res Toxicol 8: 166- 181).

EXAMPLE III

TMAuria and variation of theFM03 Gene

Dietary and Pharmacogenomic Implications

The relationships between choline (precursor of trimethylamine (TMA)), TMA and choline products (phosphatidylcholine, acetylcholine) in individuals with trimethylaminuria (TMAuria, OMIM 602079) and normal controls may be characterized to determine the optimum dietary treatment and choline requirements for individuals with TMAuria. Patients with TMAuria may be treated with riboflavin (co-factor). The consequences of polymorphic variants of theFM03 gene for TMA metabolism may be understood and applied the metabolism of other nitrogen-containing FM03 substrates and common medications.

Treatment of individuals with TMAuria

Individuals with TMAuria may be supplemented with riboflavin. The vitamin riboflavin (vitamin B2) is the cofactor for the FMO FAD-dependent enzyme family. Supplementation with riboflavin may increase the residual activity of FM03 and other FMO isoforms known to oxygenate TMA as observed in other disorders.

FMO enzymes are known to be inducible by substrate loads and hormones (Cashman J (1995) Chem Res Toxicol 8: 166-181). Affected probands may be studied. The

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patients may take a once daily dose of riboflavin 200 mg for a period of two months. A baseline urine sample for TMA and TMA N-oxide (FAB-MS) may be measured and at intervals of 4,6 and 8 weeks for measurement of effect.

The three FM03 polymorphisms(E158K, V257M and E308G) may be characterized further. These polymorphisms may exert a clinical effect on TMA oxygenation, applicable perhaps to other FM03 substrates including commonly used medication. DNA samples from 70 extended French Canadian families (450 individuals) from the Chicoutimi region of Quebec; (Hypertension sib-pair analysis study). have been genotyped for the three polymorphisms, and approximately 20% of individuals are homozygous or compound heterozygous for one or more of these polymorphisms. Individuals with these genotypes may exhibit a difference in TMA oxidation (used as a marker of decreased N-oxygenation). A rapid FAB-MS assay for TMA and its N-oxide may be used to assess TMA and TMANO in the informative cases. A detailed drug history may also be taken from these individuals to assess whether they are taking other medications likely to be FM03 substrates for future pharmacogenetic studies.

From a large cohort of individuals with TMAuria worldwide (confirmed cases in North America and Australians) plasma choline, acetylcholine, phosphatidylcholine, and TMA and TMANO may be measured in urine in controls (adult and pediatric). This may also be performed for individuals with untreated TMAuria and individuals on a low choline diet. Dietary evaluation of daily choline intake (free choline, lecithin, phosphocholine, phosphatidylcholine) may be analyzed using a 3-day dietary recall for the days

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prior to the blood test. The choline content of foods may be analyzed and correlated with TMA oxidation results and genotype. As choline is oxidized to betaine, important in homocysteine remethylation, total homocysteine may also be measured in individuals on a choline-restricted diet. TMA and its N-oxide may be measured using the stable isotope FAB method using a 5 cc urine aliquot. 15N TMA and TMANO may be used as internal standards. Choline, phosphatidylcholine and acetylcholine may be measured from a blood sample extract using electrospray tandem mass spectrometry.

Measurements of urinary TMA and its N-oxide may be used also to determine the riboflavin effect and the in vivo effects of polymorphisms, as mentioned above.

Statistical analysis

From data derived from frequency of genotypes as illustrated in Table 7, the number of individuals that will have informative genotypes may be estimated (presence of greater than 2 polymorphisms (cis or trans)) ; 2 variant alleles:(30%), 3: (14.3%), 4: (2.5 or more: (less than 1%). The estimated sample size of 76 (allowing for non-compliance) may permit adequate comparison between cases and controls.

TheStudent's t test may be used to test the hypothesis that the group of individuals with variant genotypes have a significantly lower mean percentage of N-oxidation than controls. If the sample size permits, the study group may be divided into those that carry 2,3 or 4 polymorphic alleles and the t- test performed on the distribution of N-oxygenation for each of these categories compared to the central distribution.

These studies may improve our understanding and treatment ofTMAuria and our understanding of the

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medical relevance of the polymorphisms for TMA metabolism applicable to other FMO substrates.

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Table 7 Genotypes of Possible Pharmacogenetic Significance
Observed in Chicoutimi Region
EMI37.1

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 <tb> Polymorphisms <SEP> Allele1 <SEP> Allele2
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 1 designates wild allele, 2 designates variant allele

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EXAMPLE IV Polymorphisms of the flavin containing monooxygenase 3(FM03) gene as a risk factor for essential hypertension in French Canadian
 Interindividual variation in blood pressure is genetically determined resulting from the interaction of susceptibility alleles and environmental exposures.

We previously documented that the rare mutations of the FM03 gene (a phase 1 xenobiotic detoxicating gene) cause the inborn error of metabolism Trimethylaminuria and may predispose affected individuals to essential hypertension (HBP) and abnormal

catecholamine polymorphisms(E158L (c. 488 G-A), E308G (c. 923 A-G) and V257M (c. 769 G-A)) and haplotypes were phased in 70 extended families from Quebec. Of 8 possible haplotypes, 6 were observed. These in vivo and in vitro data indicate that these polymorphisms in particular haplotypes confer functional pharmacogenetic effects.

In accordance with the present invention, now is reported a case control study of the association between variant FM03 haplotypes and early onset HBP (< 55 years) in a Quebec French Canadian population. The distribution of haplotypes was determined between (1) HBP propositi(n = 60), (2) HBP relatives(n = 229), (3) non-HBP control relatives (n= 145) and (4) unrelated non-HBP French Canadian controls(n = 133).

There was a significant difference noted between the distribution of haplotypes between groups (1) and (4) ($p = 0.026$). The presence of one variant haplotype in individuals conferred a relative risk of HBP of 1.22 variant haplotypes. These data suggest that the above common polymorphisms of theFM03 gene in the presence of adverse environmental exposures may increase the risk of individuals in Quebec to early onset HBP.

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While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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